REMARKS

The invention relates to methods for generating a population of variant DNA molecules in bacterial cells using homologous recombination, the method being generally referred to as directed gene assembly.

Claims 1-36 and 45-59 are pending in the present application. Claims 37-44 have been canceled.

Claims 1, 13 and 16 are currently amended. Claims 1 and 13 have been amended to clarify that a population of bacterial cells in which homologous recombination has occurred are selected for in the recited method. Support for this amendment is found in the as-filed specification on page 13, lines 13 to 29, and in the Examples on page 55, lines 26 to 33, among others. Claim 16 has been amended to replace the phrase "non-replicating plasmid" with "suicide vector", the latter of which has proper support in the original claims and in the specification on page 7, lines 7 to 11, referring to Figure 10. Thus, no new matter has been added by way of these amendments to the claims.

In the Specification:

The Examiner has objected to the specification as failing to provide proper antecedent basis for the claimed subject matter. Specifically, the Examiner states that the phrase "wherein the donor vector is a non-replicating plasmid" in claim 16 is not mentioned in the specification and should be amended to recite the phrase "wherein the donor vector is a suicide vector." Claim 16 has been amended according to the Examiner's suggestion.

The Examiner has also objected to the specification due to the presence of hyperlinks. Applicant has amended the specification to remove the "http://" portion of the link, thereby deleting the hyperlinks at each occurrence.

Rejection of claims 1, 4, 8-10, 12-13, 15, 19-21, 35-36, 45 and 58-59 pursuant to 35 U.S.C. §102(b)

Claims 1, 4, 8-10, 12-13, 15, 19-21, 35-36, 45 and 58-59 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Hartley (US Patent No. 5,888,732). Specifically, the Examiner asserts that Hartley discloses a method for recombinational cloning whereby a donor vector comprising a gene flanked by two recombination sites and a target

vector comprising a gene, which can be a positive or negative selectable marker, is flanked by two recombination sites.

It is hornbook law that "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." MPEP §2131 (quoting *Verdegaal Bros. v. Union Oil Co. of Calif.*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987)). "The identical invention must be shown in as complete detail as is contained in the . . . claim." *Id.* (quoting *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989) (emphasis added). Therefore, Hartley must describe each and every element of claims 1, 4, 8-10, 12-13, 15, 19-21, 35-36, 45 and 58-59 in order to anticipate these claims under 35 U.S.C. §102(b), and this reference does not satisfy this requirement.

Applicant respectfully submits that Hartley does not teach each and every element of claims 1, 13 or those that depend therefrom. Applicant has amended claims 1 and 13 to specify that the population of bacterial cells which are selected for are those which have undergone homologous recombination. Thus, the population of bacterial cells which are selected for are those which have undergone homologous recombination, not site specific recombination as disclosed in Hartley. In addition, at least one of the first donor sequence and first target sequence, or the second donor sequence and second target sequence are not identical to each other.

Hartley relates to methods of utilizing site specific recombination to generate chimeric molecules with desired characteristics. Specifically, Hartley discloses utilization of the cre/lox system or the integrase/att system of site specific recombination to generate these molecules. The cre/lox system utilizes 34 base pair loxP sequences as the locus of the crossover during recombination (Hartley, column 13, lines 63-67). As is known to one of ordinary skill in the art, cre recombinase will only mediate recombination between the loxP sites if they are identical to each other. Thus, the cre/lox system will not function if there are any mismatches between the loxP sites. Claims 1 and 13 recite that at least one of the donor/target pairs are not identical to each other. As such, the cre/lox system would not work according to the method of claims 1 or 13.

Hartley also discloses the use of the integrase/att system of site specific recombination. This integrative recombination system involves the use of attP (240 bp) and attB (25 bp) sites whereby recombination with target att sites results in the formation of two new

sites: attL and attR (Hartley, column 14, lines 26-28). Each att site contains a 15 bp core sequence and in order for efficient recombination to occur between the attP and attB sites, the 15 bp core sequences must be identical (Hartley, column 14, lines 44-54). Hartley requires that in the att system, a length of 15 bp between donor and target is identical to each other. In contrast, the present claims do not require a minimum stretch of identical base pairing between each donor and target.

Further, the differences between homologous recombination and site specific recombination were well known to the skilled artisan at the time of filing of the present application. This is evidenced in the disclosure of the well known textbook "Molecular Cell Biology," Fourth Edition, by Lodish et al., published in 1999. A copy of pages 488 and 489, as well as the identifying information for the textbook are included herewith for the Examiner's convenience. As is apparent at the bottom of page 488 and top of page 489, Lodish et al. in 1999, considered homologous recombination to be different from site-specific recombination. As such, the site-specific recombination disclosed in Hartley cannot anticipate the homologous recombination as disclosed and claimed by Applicant.

Therefore, Hartley does not anticipate the present invention because it does not disclose each and every element of the claimed invention. Applicant respectfully requests reconsideration and withdrawal of this rejection.

Claims 2, 3, 5-7, 11, 14, 16-18, 22-34 and 46-57

In the Office Action, the Examiner has objected to claims 2, 3, 5-7, 11, 14, 16-18, 22-34 and 46-57 as being dependent upon a rejected base claim and states that these claims would be allowable if re-written in independent form. It is Applicant's view that these claims are presently allowable given the amendments to the base claims from which they depend and such action is respectfully requested.

Summary

Applicant respectfully submits that the objections to the specification and the rejection of the claims under 35 U.S.C. §102(b) have been overcome, and that claims 1-36 and 45-59 are now in condition for allowance. Reconsideration and allowance of these claims is respectfully requested at the earliest possible date.

Respectfully submitted,

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(Date)

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Enclosures: Petition for a three month extension of time
Copy of Revocation and Power of Attorney
Copy of pages 488-489 of Molecular Cell Biology

FOURTH EDITION

MOLECULAR CELL BIOLOGY

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S. Lawrence Zipursky
Paul Matsudaira
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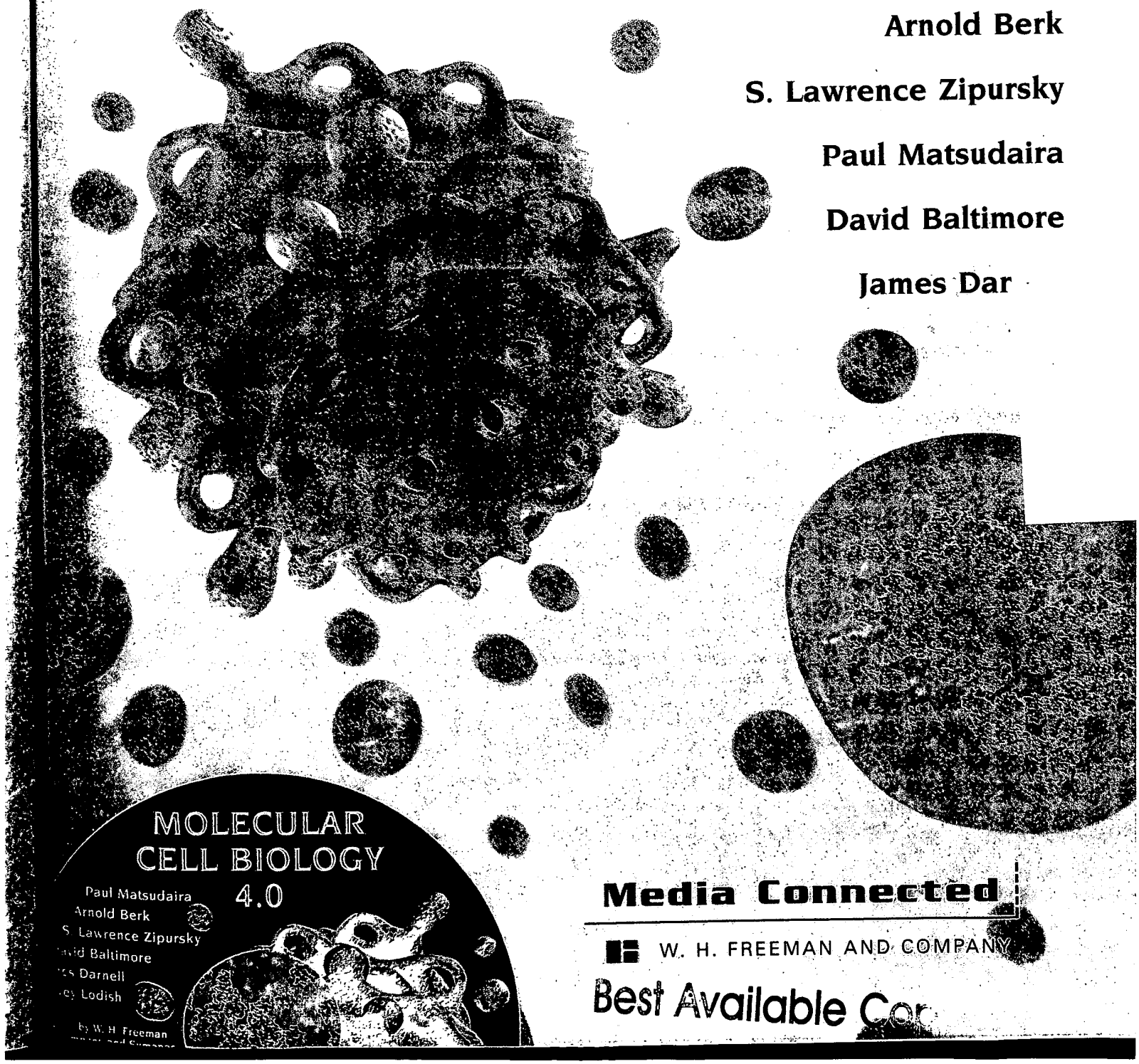

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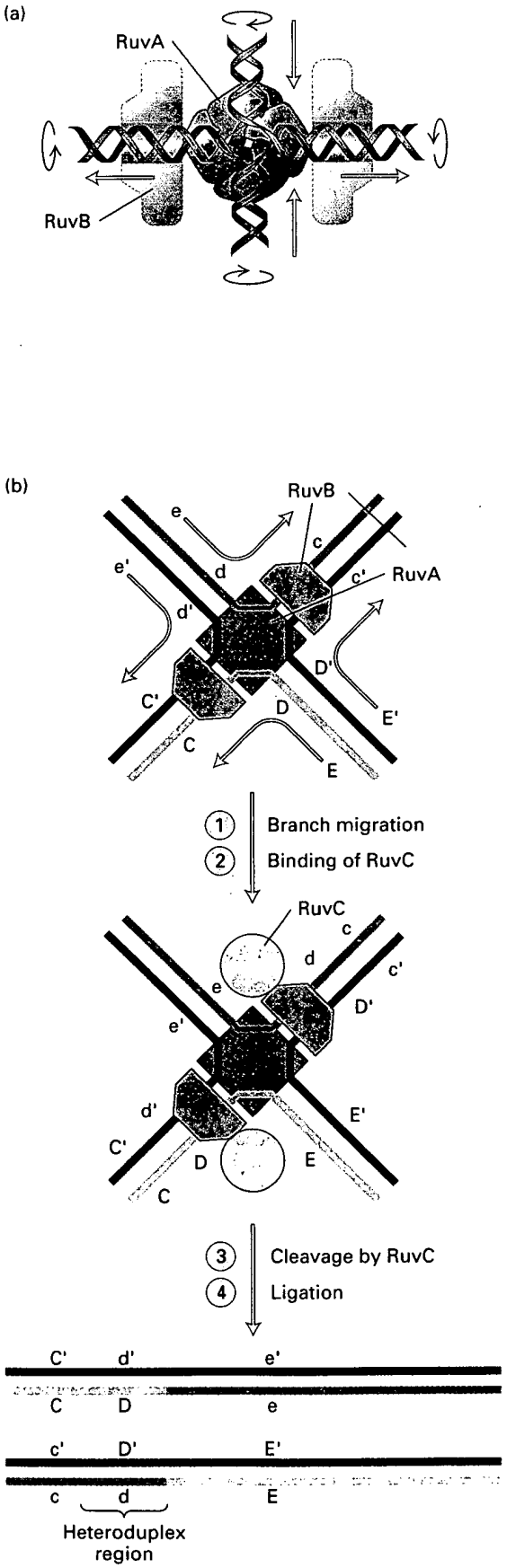

◀ **FIGURE 12-35 Action of *E. coli* proteins in branch migration and resolution of Holliday junctions.** (a) Model of the association of RuvA and RuvB with a Holliday junction as determined by electron microscopy. For clarity the proteins are drawn behind the DNA; in reality the DNA passes through the central hole of each RuvB hexamer. Powered by ATP hydrolysis, the ringlike RuvB molecules rotate the DNA double helices inside them, much in the way a screw is rotated inside a nut. The two RuvB proteins impart equal and opposite rotational forces to the DNA, as indicated by the black circular arrows. The straight arrows indicate the direction of movement of DNA through the RuvA/RuvB complex. (b) Diagram illustrating migration and resolution of a Holliday junction. (Alleles and complementary strands are indicated as in Figure 12-31.) Movement of a junction through the RuvA/RuvB complex generates heteroduplex regions, exactly as observed in the experiment shown in Figure 12-34. Following branch migration ①, two molecules of the RuvC endonuclease bind to RuvA ② and cut the intermediate at two points that are 180° apart ③. Ligation of the cut ends completes resolution ④. In this example, two recombinant molecules are formed, but if RuvC cleaved at the two other points, nonrecombinant DNAs would be produced (see Figure 12-31). [Adapted from S. C. West, 1996, *Cell* **86**:177; J. Raftery et al., 1996, *Science* **274**:415; and A. Kuzminov, 1996, *BioEssays* **18**:757.]

RuvB complex and then cut the DNA intermediate at two sites 180° apart; subsequent ligation generates recombinant (or nonrecombinant) molecules containing a segment of heteroduplex DNA (Figure 12-35b).

Homologous Eukaryotic Recombination Enzymes Although RecA, RecBCD, and RuvA, B, and C were initially identified in *E. coli,* all eukaryotic cells, including human cells, produce proteins of similar structure and function. For instance, the human and yeast RAD51 proteins, which are homologous in sequence, catalyze pairing of homologous DNA segments and DNA strand insertion similarly to RecA. A Topo II–like protein encoded by the yeast *Spo11* gene generates the double-strand breaks that occur during meiotic recombination, and homologous proteins are found in bacterial and other eukaryotic cells. Thus the molecular mechanism of homologous recombination most likely is similar in all types of cells.

Cre Protein and Other Recombinases Catalyze Site-Specific Recombination

Homologous recombination occurs randomly between two homologous DNA segments, and there is relatively little specificity as to the site at which the actual crossover occurs. In *site-specific* recombination, a different type of process, relatively short, unique nucleotide sequences in two DNA molecules are recognized by enzymes called *recombinases,* which then catalyze the joining of the two molecules. Several examples of site-specific recombination have been discovered in both prokaryotic and eukaryotic cells.

One well-studied example, the integration of bacteriophage λ into a particular site in the *E. coli* chromosome (see Figure 6-19), is catalyzed by a viral enzyme called *integrase.* The genome of λ phage contains a 15-bp attachment site whose 7-bp core sequence is identical with the integration (or attachment) site in the host-cell DNA. Integration can be carried out in a cell-free reaction system with only two purified proteins, λ integrase and integration host factor, a cellular protein. Integrase also catalyzes the reverse reaction, excision of the circular λ phage DNA from a bacterial chromosome.

The site-specific recombination reaction that is best understood at the molecular level is catalyzed by Cre, a protein encoded in the genome of bacteriophage P1. During phage P1 DNA replication, long multimeric DNAs are produced; these are resolved into monomeric P1 DNAs by recombination at loxP sites, which separate the P1 DNA monomers composing a multimeric DNA (Figure 12-36).

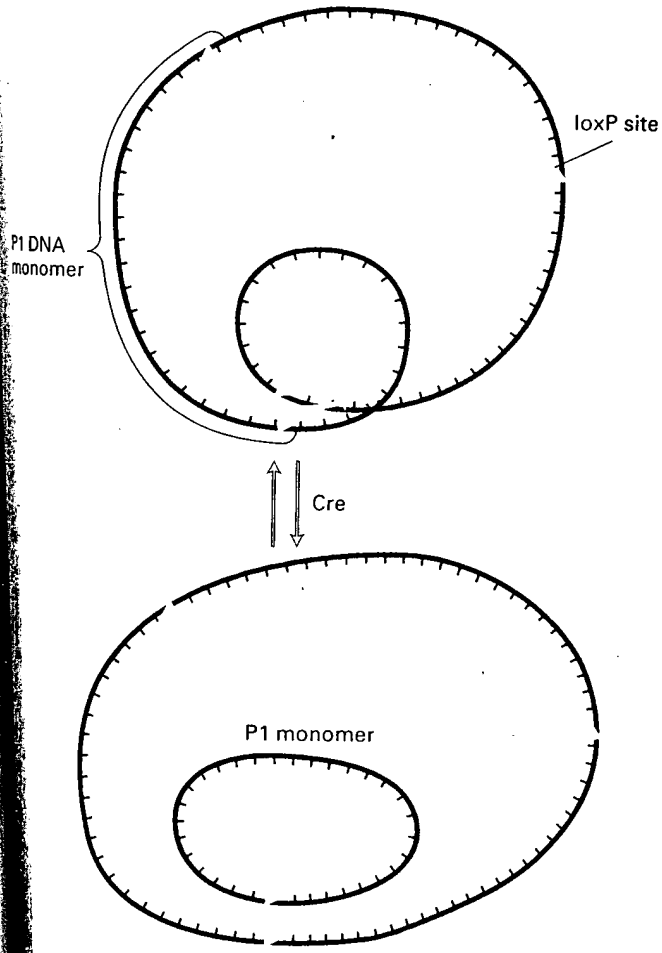

FIGURE 12-36 Cre protein, encoded by phage P1, catalyzes site-specific recombination between loxP sites on multimeric copies of P1 DNA, generating a circular monomeric DNA. Repetition of this process eventually converts the entire multimeric DNA into circular P1 monomers.

The Cre protein that catalyzes this reaction is similar in structure and function to λ integrase. Moreover, the excision of monomeric P1 DNAs from a multimeric circle by Cre is mechanistically similar to the excision of λ DNA from the bacterial chromosome by integrase.

The mechanism of Cre-catalyzed recombination is depicted in Figure 12-37. This mechanism involves the sequential formation of two transient intermediates in which Cre and DNA are covalently linked by phosphotyrosine bonds, similar to those between DNA and topoisomerase II (see Figure 12-14, *inset*). Reaction of the first Cre-DNA intermediate generates a Holliday-type structure; reaction of the second one yields the recombinant double-stranded DNA products. By using DNA molecules with mutations or single-strand breaks in the short loxP homologous recognition sites, researchers have been able to stop the Cre-catalyzed reaction at several stages and collect intermediates (Figure 12-38).

The P1 recombinase system has proved useful to mouse geneticists. Because many genes are required at multiple stages of development, "classical" gene-knockout mice, produced by the procedure depicted in Figure 8-34, frequently die as early embryos. However, the P1 loxP-Cre recombinase system has enabled geneticists to generate animals in which a particular gene of interest is deleted only in one specific tissue (see Figure 8-35). Such tissue-specific knockout mice enable researchers to study the function of any gene in just one tissue of the adult animal. Thus, an understanding of how one site-specific recombination system excises a DNA segment out of a specific chromosomal site led to the development of an important tool for mouse geneticists.

SUMMARY Recombination between Homologous DNA Sites

- During homologous recombination, two duplex DNA molecules are broken and strands are exchanged. This process, which occurs randomly along the genomes of all organisms, plays an important role in generating genetic diversity.
- Double-strand breaks in DNA initiate most cases of homologous recombination. The break becomes enlarged to gaps, forming single-stranded 3′ recombinogenic ends that invade the other duplex. Repair synthesis of the missing regions forms an intermediate containing two crossed-strand Holliday junctions. Resolution of this intermediate occurs by rotation followed by cleavage and ligation of two strands at each Holliday junction (see Figure 12-31).
- In *E. coli,* a recombinogenic end created by the RecBCD enzyme complex is stabilized by binding of RecA protein (see Figure 12-32). Catalyzed by RecA, the single-stranded 3′ end then pairs with and invades a homologous duplex DNA segment, forming an intermediate containing two regions of heteroduplex DNA